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TITLE: Evaluation of Altered Stromal/Epithelial Tissue Arrangement of the c-Kit Messaging System in the Control of Breast Cancer

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## **Introduction**

The purpose of this research is to evaluate stromal influence on breast cancer growth particularly the role of c-Kit expression in the stroma on the growth and migration of breast cancer cells. The model is based on obtaining breast cancer cells and stromal cells from the same patient, growing these cells in an organotypic environment in culture and in immune deficient mice, and studying the influence of c-Kit modulation on tumor behavior.

## **Body**

The Hs578T breast cancer and its surrounding “normal” fibroblasts (Hs578Bst) were isolated and established as cell lines by AJ Hackett in 1977. We obtained these cells from the American Type Culture Collection and established in our hands their growth properties in culture.

Growth in Culture. The doubling time of Hs578T is 48 hours and the line is immortalized showing no senescence. The doubling time of Hs578Bst is 96 hours, it is not immortalized, and begins to senesce after 6 generations. The slow doubling time of the fibroblast line and the need to continuously re-establish it in culture from frozen stocks has slowed progress on completion of some of the proposed experiments.

Sensitivity to Gleevec and Alpha-fetoprotein derived peptide (AFPep). Gleevec and AFPep have both been shown to inhibit c-Kit. Their effect on the above mentioned cell lines was not known. We found the  $IC_{50}$  of Gleevec on Hs578Bst and Hs578T to be  $1.4 \times 10^{-5}$  M and  $1.4 \times 10^{-5}$  M, respectively. The  $IC_{50}$  of AFPep on Hs578Bst was  $1.0 \times 10^{-5}$  M, whereas on Hs578T it was non-growth inhibitory. (Fig. 1A-D)

Interaction of Hs578T with Hs578Bst in co-culture as measured by confocal microscopy. Hs578T was labeled with a red fluorescent dye (CellTracker™ Red CMTPX, Molecular Probes) and Hs578Bst was labeled with a green fluorescent dye (CellTracker™ Green BODIPY®, Molecular Probes). The fibroblasts were suspended in collagen type I and layered onto Mat Tek dishes. The collagen layer was approximately 100µm in thickness. Maintaining a uniform and flat collagen layer was a technical problem that took some time to work out. This was important for achieving uniform depth measurements in this model. The fibroblasts grown in this collagen layer were elongated and spread out (Fig. 2A). When tumor cells were placed on top of the collagen layer devoid of fibroblasts, the tumors were also elongated and spread out with projections that formed networks between the cells (Fig. 2B). Interestingly, when tumor cells were placed on a fibroblast-containing collagen layer, both tumor and fibroblasts were rounded with no projections or networks between cells (Fig. 2C). This contextual cytoarchitectural change in morphology certainly requires further exploration. The rounding up of both cell types occurred within the first 24 hours after their co-culture (Fig. 2D, side view of co-culture). This morphology maintained itself at later time points (48-72 hours) during which time there was clear evidence of tumor migration down into the fibroblast layer (Fig. 2E, side view of co-culture). Future experiments in this system will evaluate tumor morphology and migration in the presence of normal fibroblasts treated with either Gleevec or AFPep and c-Kit overexpressing fibroblasts treated with either Gleevec or AFPep.

c-Kit overexpression. We have developed both plasmid and retroviral based expression constructs for c-Kit. In brief, c-Kit was PCR amplified from the pBluescriptR plasmid containing full-length c-Kit gene. The resulting PCR product was cloned into *pCR2.1-TOPO* cloning vector and confirmed by sequencing. This plasmid was then used to insert the c-Kit cDNA into plasmid (pCDNA3/c-Kit) and retroviral (pBABEpuro/c-Kit) expression constructs. The pCDNA3/c-Kit and pBABEpuro/c-Kit have been used to either transfect or infect Hs578Bst

fibroblasts. We are currently in the process of confirming transgene expression by immunoblotting for c-Kit. Immunoblots of c-Kit in basal Hs578T and Hs578Bst are shown in Fig. 3.

**Tumorigenicity.** Hs578T human breast cancer cells have been reported to be non-tumorigenic in immune deficient mice (ATCC web site). We felt we could overcome that obstacle with strategies typically used in our laboratory to grow freshly resected human tumors. We validated that these cells did not grow when transplanted either subcutaneously or into the mammary fat pad (mfp) of severe combined immune deficient (SCID) or athymic nude mice. We solidified these cells in a fibrin clot and transplanted them into the mfp or under the kidney capsule of SCID mice and found no significant tumor growth. We admixed these cells with matrigel, transplanted them into the mfp of SCID mice and obtained within 14 days nodules that measured 1mm to 2mm in diameter but these nodules did not progress in tumor growth. We admixed Hs578T tumor with Hs578Bst fibroblasts in matrigel and found tumor take and tumor outgrowth in 100% of the mice within 7 days and progressive tumor growth thereafter (Fig. 4), clearly demonstrating that the presence of fibroblasts promoted tumor growth. This experiment was repeated to determine the effect of Gleevec on tumor take and tumor growth. Gleevec had no effect on tumor take but did significantly inhibit tumor growth (Fig. 5). **Remaining to be done:** Evaluate the effect of AFP derived peptide on tumor take and tumor growth of this admixture of cells. Evaluate tumor take and tumor outgrowth when Hs578T tumor is admixed with Hs578Bst fibroblasts which are overexpressing c-Kit and evaluate effect of Gleevec and AFPep on the tumor take and tumor growth of this admixture of cells.

Tumor was labeled through stable infection with green fluorescent protein (GFP), injected with fibroblasts into the mfp of SCID mice, and fluorescence was followed using the Kodak Image Station 4000 MM Multimodal Imaging System. As shown in Fig. 6, tumor was detectable by fluorescence before it was palpable (small tumor); and fluorescence provided a pattern of growth that yielded more detail than that achievable by palpation (large tumor). This needs further study with tumor admixed with c-Kit-overexpressing fibroblasts.

**Proliferation in culture as measured by FACS.** Hs578T was labeled with a green fluorescent dye (CellTracker™ CFSE Cell Proliferation Kit, Molecular Probes) and was admixed with unlabeled Hs578Bst which were then plated on plastic 35mm dishes. Cells were lifted off the plates daily over the course of a week and were fixed with paraformaldehyde. The cell solution was subjected to FLOW cytometry from which approximate cell number of Hs578T was determined based on number of events recorded. Preliminary data suggest that more proliferation of tumor occurs in the presence of fibroblasts, but this will be studied further in future experiments. However, these preliminary data are very intriguing especially in light of the in vivo findings that Hs578T is more tumorigenic when in the presence of its corresponding fibroblasts.

### **Key Research Accomplishments**

1. The technology for viewing in three dimensional format the morphology, growth, and migration of breast cancer cells within the context of surrounding stromal cells has been established.
2. The sensitivity of human breast cancer cells and their surrounding fibroblasts to growth inhibition by Gleevec and AFP-derived peptide (both inhibitors of c-Kit) has been established.
3. c-Kit expression in human breast cancer cells and in its surrounding fibroblasts has been determined.
4. c-Kit has been overexpressed in the fibroblasts surrounding a human breast cancer.

5. The human breast cancer plus or minus its surrounding fibroblasts has been grown in immune deficient mice and its sensitivity to Gleevec has been determined.
6. The influence of c-Kit overexpressing fibroblasts on the growth and migration of breast cancer remains to be determined, as does fibroblasts in which c-Kit is inhibited.

### **Reportable Outcomes**

1. Growth rates of Hs578T (human breast cancer) and Hs578Bst (surrounding fibroblasts) are 48 hours and 96 hours doubling time, respectively.
2. The morphology and migration of Hs578T is changed when it is grown in the presence of its surrounding fibroblasts.
3. The tumorigenicity of Hs578T in SCID mice is significantly enhanced when it is transplanted in the presence of its surrounding fibroblasts.
4. Gleevec significantly inhibits the growth of Hs578T xenografts.
5. Gleevec significantly inhibits the growth of Hs578T and Hs578Bst in culture, whereas AFPep only inhibits the growth of Hs578Bst in culture.

### **Conclusion**

As described above the work is not yet complete, and a no cost extension of this award has been requested. The reasons for the no cost extension are detailed in a letter to Ms. Lisa Trott (the contract specialist for this award), and the letter is included as an appendix to this report. We are enthused about the progress that has been made on this study of stromal regulation of breast cancer growth and hope that the DOD-BCRP will grant this no cost extension and allow us to complete the work that was originally proposed.

### **List of Personnel**

The personnel involved in completing the work are:

James A. Bennett, PhD

Andres Melendez, PhD

Nicole Lostritto, BS

### **Bibliography**

No publications or meeting abstracts have yet been generated from this work.

## Supporting Data

Fig. 1A

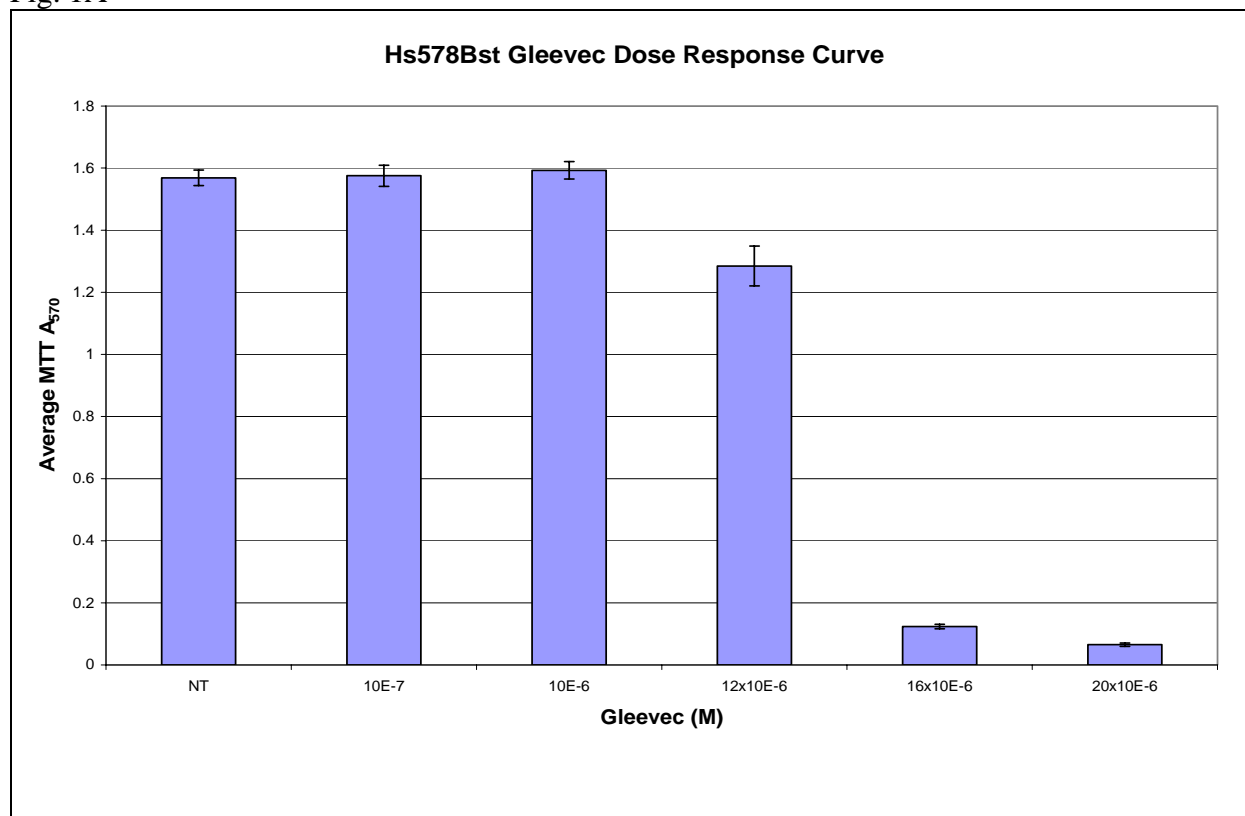


Fig. 1B

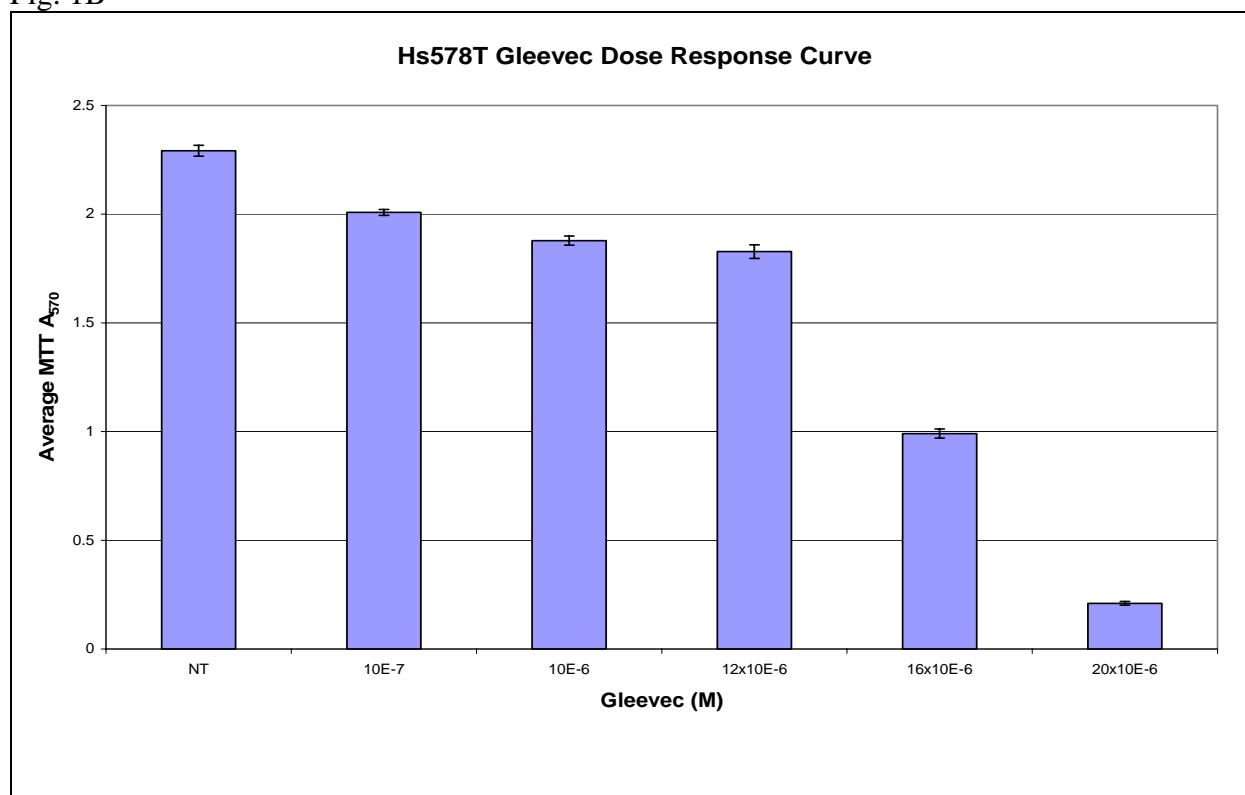


Fig. 1C

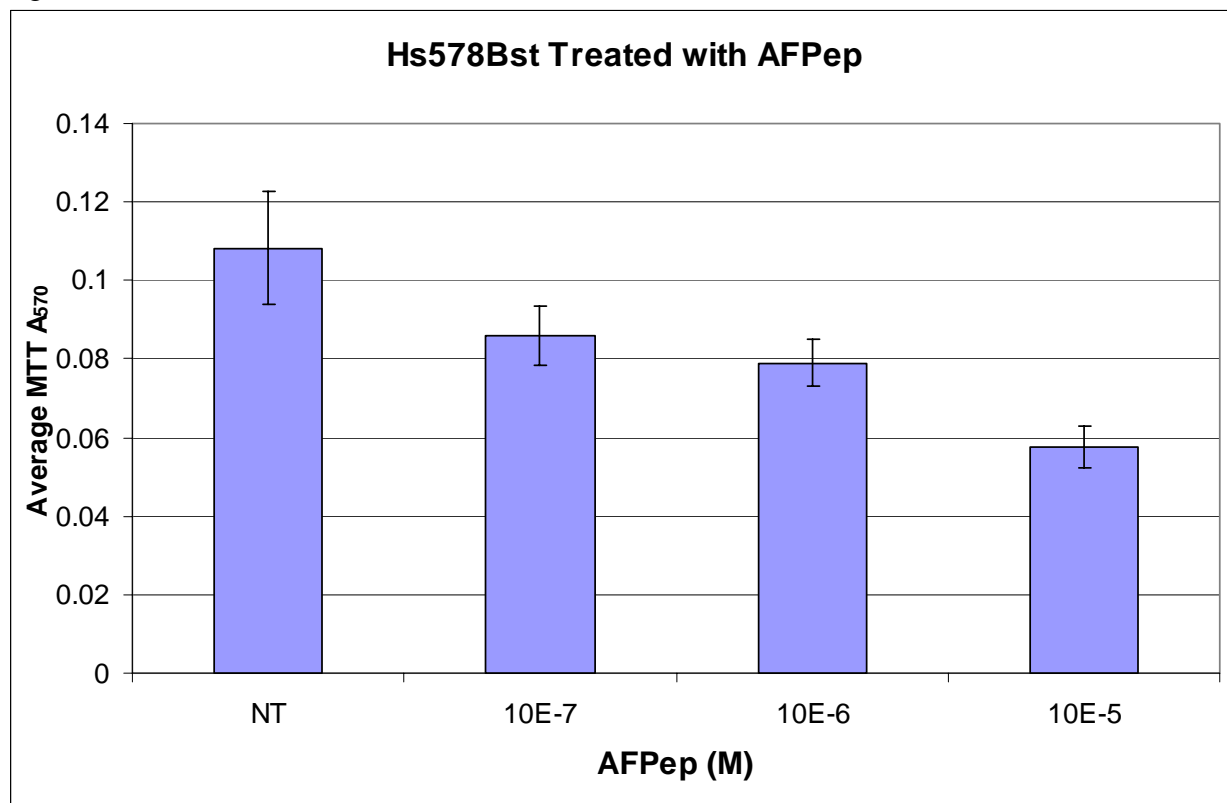


Fig. 1D

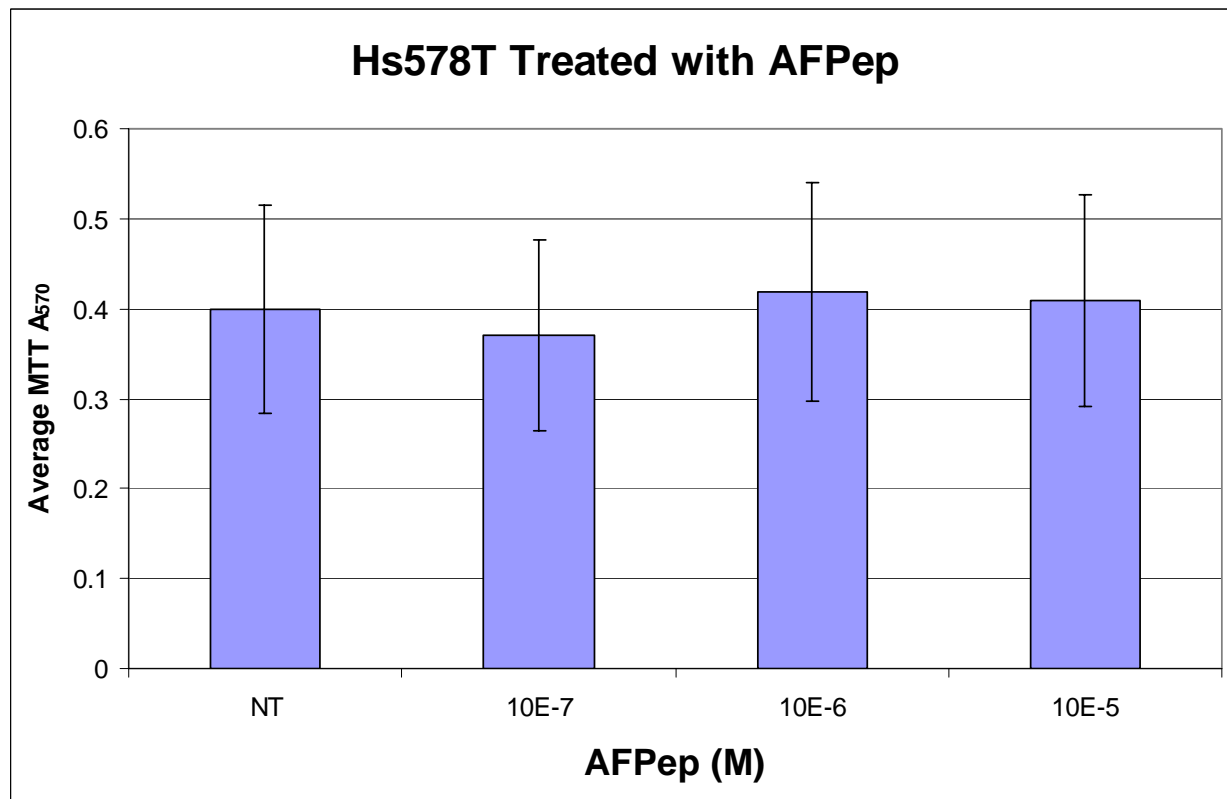




Fig. 2A Hs578Bst alone

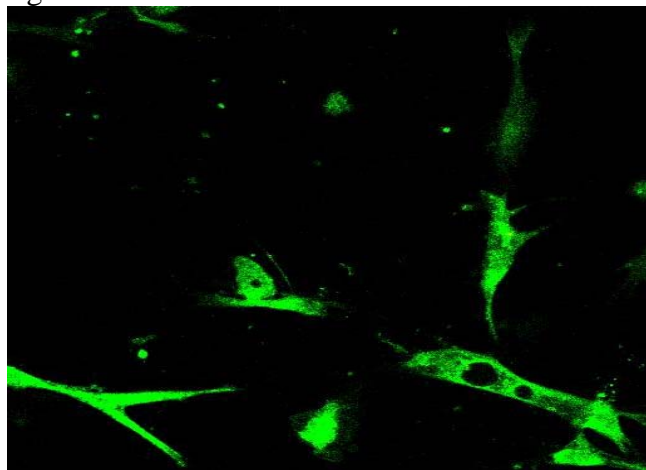


Fig. 2B Hs578T alone

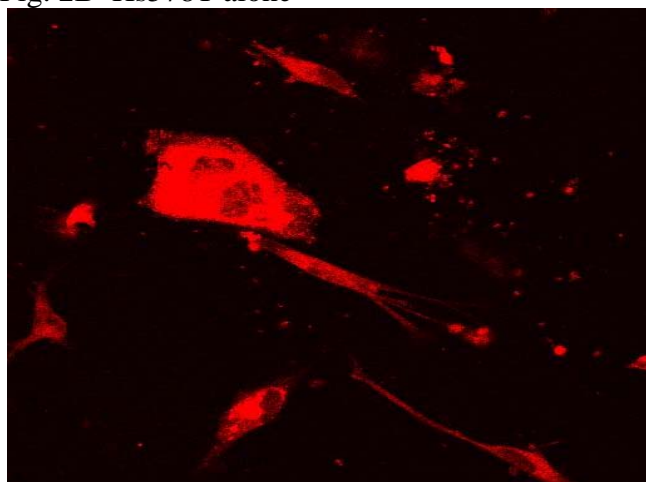


Fig. 2C Hs578Bst + Hs578T

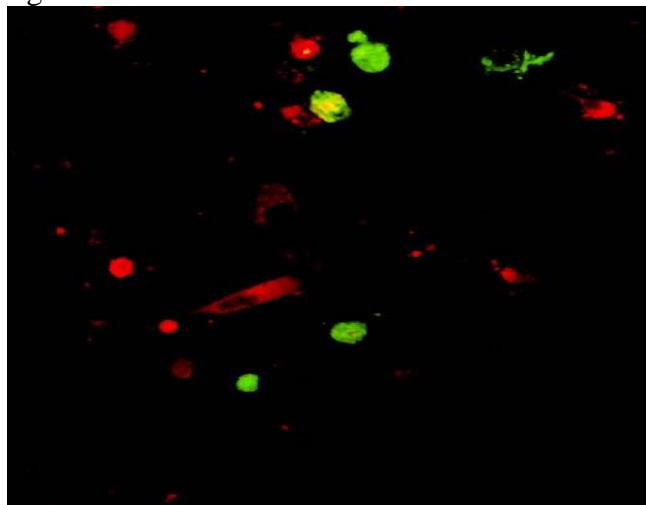


Fig. 2D Hs578Bst + Hs578T, side view first 24 hours

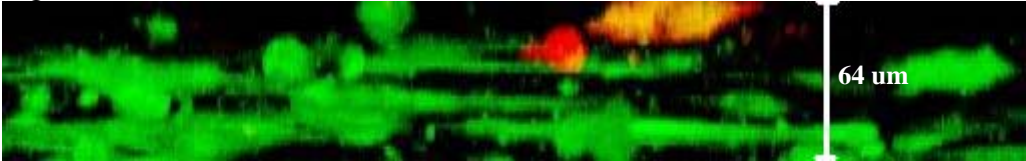


Fig. 2E Hs578Bst + Hs578T

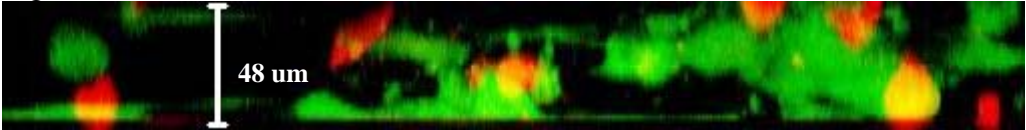


Fig. 3 Western Blot of c-Kit. Tumor (T); Fibroblast (F); Molecular weight markers (MW)

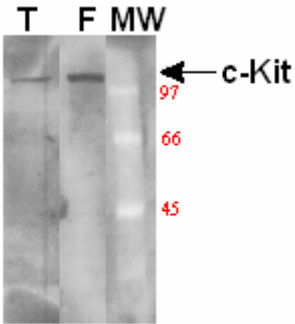


Fig. 4

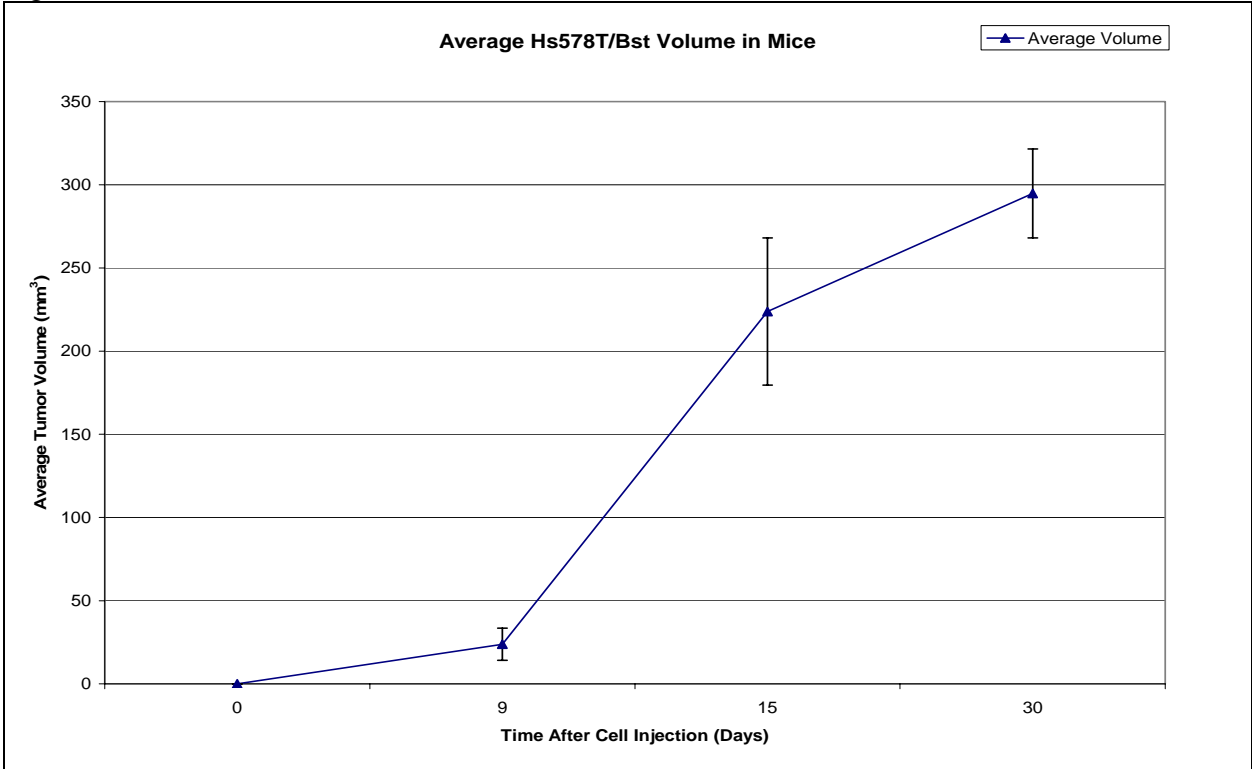


Fig. 5

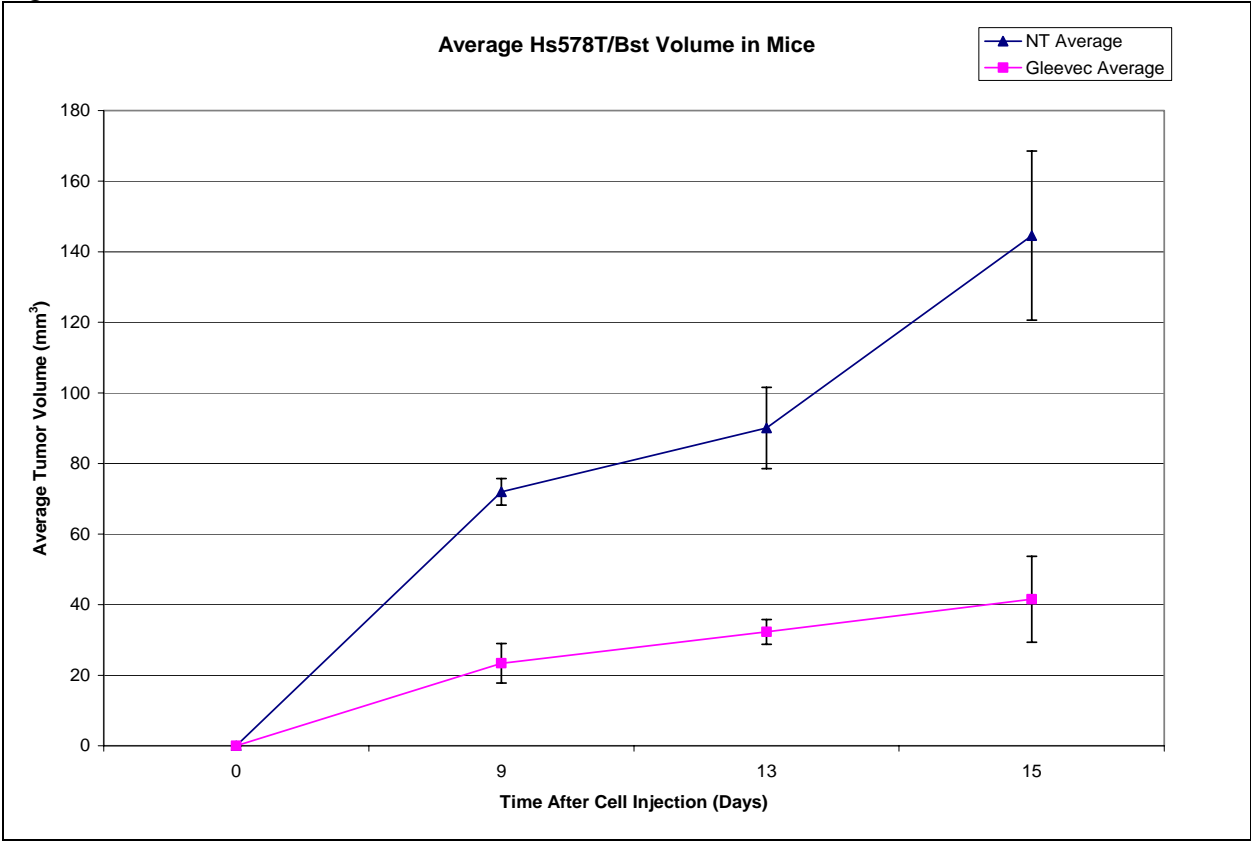
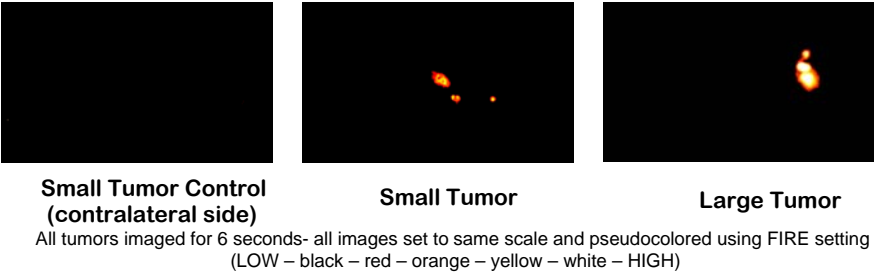


Fig. 6



White light image with large tumor overlayed

